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Award Number: DAMD17-99-1-9145

TITLE: Role of Nuclear Receptor Coregulators in Hormone Resistant Breast Cancer

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REPORT DATE: September 2000

TYPE OF REPORT: Annual Summary

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)

2. REPORT DATE
September 2000

3. REPORT TYPE AND DATES COVERED
Annual Summary (1 Sep 99 - 31 Aug 00)

4. TITLE AND SUBTITLE
Role of Nuclear Receptor Coregulators in Hormone Resistant Breast Cancer

5. FUNDING NUMBERS
DAMD17-99-1-9145

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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

Report contains color photos

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for public release; distribution unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT ***(Maximum 200 Words)***
Subject: Development of resistance to the widely used antiestrogen, tamoxifen, is a prevalent issue in breast cancer patients. The mechanisms underlying tamoxifen resistance are poorly understood and occur despite continued estrogen receptor (ER) expression. **Purpose:** To determine whether the expression levels of transcriptional coregulators, which influence ER activity, affect tamoxifen sensitivity. **Scope:** I hypothesized that decreased levels of the corepressors SMRT and N-CoR, or an increase in levels of the coactivators, SRC-1 and L7/SPA, in breast tumors, results in an increase in the partial agonist activity of tamoxifen, expressed as "resistance". **Major Findings:** I developed and optimized a sensitive and quantitative assay to measure coregulator expression levels in breast cancer cell lines and tumors. Using the assay, expression levels of the four coregulators were measured in a group of tamoxifen sensitive and resistant tumors. N-CoR and SMRT levels were overall lower in tamoxifen resistant tumors, compared with the tamoxifen sensitive group. The levels of coactivator expression did not differ among the tumors. Furthermore, ER transcriptional studies in the presence of SMRT suggest that coregulators suppress the partial agonist activity of tamoxifen by a novel mechanism. **Significance:** Statistical analysis of my results suggests that decreased levels of corepressor expression in breast tumors may predict the development of tamoxifen resistance. The coactivators SRC-1 and L7/SPA may not be involved in tamoxifen resistance. On the other hand, other coactivators that are yet to be defined may play a role in the increased partial agonist activities of mixed antagonists, and the transition to a resistant phenotype. I next plan to identify such proteins.

14. SUBJECT TERMS
Breast Cancer, hormone resistance, corepressors, coactivators, estrogen receptor, progesterone receptor, tamoxifen, antagonist, agonist, mRNA expression, transcriptional regulation

15. NUMBER OF PAGES
34

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

NSN 7540-01-280-5500

Standard Form 298 (Rev. 2-89)
Prescribed by ANSI Std. Z39-18
298-102

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Introduction

Subject:
The antiestrogen tamoxifen is an effective treatment for estrogen receptor (ER) positive breast cancers, slowing tumor growth and preventing disease recurrence, with relatively few adverse side-effects. However, many patients who initially respond to treatment, later become resistant, despite continued expression of ER and progesterone receptors (PR). Tamoxifen has both agonist and antagonist activities, which are manifested in a tissue-specific pattern. *We hypothesized that development of tamoxifen resistance was associated with an increase in the partial agonist properties of the antiestrogen in the breast, resulting in loss of growth inhibition and even inappropriate tumor stimulation.* Nuclear receptor function is modulated by transcriptional coregulators, which are present at rate-limiting levels, and either enhance or repress receptor activity. **We speculated that the relative levels of these coactivators and corepressors might determine the balance of agonist and antagonist properties of mixed antagonists such as tamoxifen and the antiprogestin RU486.** Using a mixed antagonist-biased two-hybrid screening strategy, we previously identified two such proteins: the human homolog of the nuclear receptor corepressor, N-CoR, and a novel coactivator, L7/SPA (Switch Protein for Antagonists). In transcriptional studies N-CoR suppressed the agonist properties of tamoxifen and RU486, and L7/SPA increased agonist effects.

Purpose:
I proposed to test the hypothesis that the ratio of coactivator to corepressor expression determines whether a steroid antagonist is an inhibitor or a stimulator in a specific tumor. Specifically, **I postulated that increased expression of the coactivators, SRC-1 or L7/SPA, and/or reduced expression of the corepressors, N-CoR or SMRT, results in loss of tumor endocrine responsiveness, and enhances the inappropriate partial agonist effects of tamoxifen and the antiprogestin RU486.** Such tumors would be classified as hormone "resistant".

Scope:
In preliminary experiments I developed a sensitive quantitative RT-PCR assay to measure the levels of the four coregulators in cultured breast cancer cell lines and in breast tumors. All four coregulators were detectable at low levels in cell lines and tumors. I proposed to optimize this quantitative assay (**Aim 1**), and to use it to measure coregulator levels in a cohort of breast tumors for which tamoxifen sensitivity was known (**Aim 2**). I also proposed to use transcriptional regulation studies to determine the function and regulation of coregulators in endocrine resistant breast cancer (**Aim 3**).

The project has been carried out as described in the original Statement of Work. Substantial progress has been made in the area of all three tasks. The results obtained are outlined here according to the original task headings and subheadings.

Body

Task 1: Optimization of quantitative RT-PCR protocol. Months 1-6.

a. Optimize conditions for quantitation of L7/SPA, SRC-1, SMRT and N-CoR transcripts in breast tumors, using preliminary optimization in cell lines and limited tumor group.

Using total RNA from breast cancer cell lines, and a group of 10 breast tumor specimens, the quantitative RT-PCR assay was fully optimized. It was important to optimize conditions in both cell lines and tissue samples, since expression of the four coregulators may be different in cultured cell lines, than in breast tumors. The RNA was reverse transcribed, and 20ng of the endogenous cDNA was added to each reaction tube together with increasing duplicate concentrations of a control template (example: **Fig.1**). . The control templates used were identical to their endogenous targets, except that a restriction site had been engineered into the sequence to allow the control product to be distinguished from the endogenous one by Southern blotting. Initially a broad concentration range of control template was used with breast cancer cell line RNA. A specific control range was determined, which was predicted to amplify a product in the range of 0.1 to 10-fold of the abundance of the endogenous product, and transcripts were quantitated in all cell line RNAs **Figure 1** shows quantitations of all four coregulators in the same RNA sample, from one breast tumor specimen. The resulting bands were quantitated densitometrically, and using the known amounts of the control template in each sample, the abundance of the endogenous transcript was extrapolated. In the case of the patient shown, N-CoR transcripts were detected at 1.4 fmol per mg total RNA, SRC-1 was expressed at 0.4 fmol per mg, and L7/SPA was present at 2.1 fmol per μg total RNA.

The quantitative assay for SMRT was initially designed to distinguish full-length SMRT transcripts from a previously described alternatively spliced form, which lacks amino acids 1330 to 1375 at the C-terminus of the 1495 amino acid SMRT form (**Fig. 2A**). However, a novel second smaller alternatively spliced form was also amplified, as shown for MCF-7 cells (**Fig. 2B**). It was isolated and sequenced, and found to lack coding sequences for amino acids 1300 to 1375. This second isoform completely lacks a putative α-helical domain predicted to be functionally important in the defined thyroid/retinoic acid receptor interaction domain (RID)-2 of SMRT (**Fig.2A**). All three isoforms were detected in the cell lines (**Fig. 2C**) and tumors. SMRT expression levels in cell lines were comparable to the expression of N-CoR (**Table 1**) in the lines. In the tumor specimen shown in **Fig.1** the concentrations of the three SMRT transcripts were 0.255 (full length), 0.305 (Δ1330-1375) and 0.276 (Δ1300-1375) fmol per mg total RNA. These levels were comparable to other tumor samples, but much lower than was detected in most cell lines.

b. Complete quantitation of coregulator transcripts in cell lines and preliminary tumor group.

The quantitative RT-PCR assay was used to estimate N-CoR levels in a panel of cell lines and an initial group of ten tumors with limited follow-up information (**Table 1**). The cell lines included five breast cancer cell lines (T47Dco, T47D-Y, ZR-75-1, MCF-7 and MDA-MB-231), three "normal" breast epithelial cell lines (HBL-100, HMEC and 184) and the HeLa cervical carcinoma cell line. N-CoR transcript levels were low but detectable in all cell lines. Expression levels varied among the cell lines from 1.9 to 10.8 fmol/mg total RNA, with no correlation to the ER or PR status of the cells and no significant difference between malignant and normal cells. In the preliminary tumor group, N-CoR transcripts were detectable in eight of the ten tumors. Mean expression of N-CoR was lower in tumors than in the cell lines and was not correlated to ER or PR. The variability of SMRT expression among cell lines was not correlated to receptor status or malignancy. The strongest expression of a full length SMRT transcript was seen in the PR+ ZR-75-1 breast cancer cell line, whereas highest expression of the two alternatively spliced forms was seen in the HBL100 normal breast epithelial line.

The coactivators SRC-1 and L7/SPA were also quantitated in the cell lines and the initial tumor group (**Table 1**). Expression of SRC-1 was detectable in all samples, but was low (< 1 fmol/mg total RNA) in both cell lines and tumors. Furthermore, the expression level was not correlated to receptor status or malignant transformation. In contrast, transcripts for L7/SPA were expressed at a markedly higher level, particularly in malignant cell lines but also in "normal" cells and in the tumors. Interestingly, L7/SPA expression was significantly higher in the malignant cell lines (16 to 54 fmol/μg total RNA) than in the three "normal" breast epithelial lines (<5 fmol/μg, t-test, $p < 0.01$). In the ten tumors L7/SPA expression fell into a range similar to that of "normal" breast cells (0.4 to 5.4 fmol/μg). Of interest was the preliminary observation that L7/SPA expression is significantly higher in ER+/PR+ tumors, than in ER-/PR+ and ER-/PR- tumors (t-test, $p < 0.01$). This was further investigated, including a larger tumor group, and is discussed under the ***task 2*** sub-heading.

Task 2: Quantitative analysis of coregulator expression in tamoxifen sensitive and resistant tumors. Months 6-18.

a. Quantitate coregulator transcripts in tumors from tamoxifen sensitive and resistant tumors. N-CoR and SMRT transcript levels were quantitated in a group of breast tumors (obtained from the San Antonio Breast Tumor Bank) for which tamoxifen responsiveness was known. The group comprised five tumors from patients who had responded to tamoxifen with no disease relapse two to five years after the completion of tamoxifen treatment, and 18 tamoxifen resistant tumors from patients who had relapsed while receiving tamoxifen or within two to six months after the cessation of treatment. Transcripts for N-CoR and SMRT were detectable in all tumors. SMRT transcript levels ranged from 0.04 fmol/mg total RNA to 0.89 fmol/mg; N-CoR expression ranged from 0.0008 fmol/mg to 1.65 fmol/mg. SMRT and N-CoR expression levels were not correlated to receptor status, nodal status or tumor size. The mean expression levels of all three SMRT transcripts and of N-CoR were decreased in the tamoxifen resistant group, compared to the tamoxifen sensitive tumors (**Fig.3**). Mean levels of full-length, Δ1330-1375 and Δ1300-1375 SMRT transcripts were decreased to 72%, 72% and 69% respectively, in tamoxifen resistant tumors compared to tamoxifen sensitive tumors. Similarly, mean N-CoR expression in tamoxifen resistant tumors was 62% of tamoxifen sensitive tumors. Levels of SMRT and N-CoR were highly correlated among the tumors (t-test, $p < 0.001$). That is, tumors in which SMRT was decreased also tended to have lower N-CoR, suggesting that one corepressor does not compensate for the other.

The coactivators SRC-1 and L7/SPA were also quantitated in this tumor group. The range of concentrations of SRC-1 transcripts was somewhat higher in this group of tumors than in the initial 10 tumor group, ranging from 0.2 to 2 fmol/mg total RNA. L7/SPA levels were comparable to the other tumors, ranging between 0.025 and 8.5 fmol/μg total RNA. No correlation was seen between the coactivators and tamoxifen status in these tumors (**Fig.3**) or to nodal status or tumor size. While SRC-1 levels were not correlated to receptor status, a positive correlation was seen between PR and L7/SPA. This is discussed further below.

b. Statistical analysis of coregulator data with tumor follow-up information.
Statistical analysis of the quantitative results is continuing, in preparation for submission of a manuscript. A collaboration has been established with Dr Douglas Wolf, Ph.D., biostatistician, in the Department of Obstetrics and Gynecology, University of Colorado Health Sciences Center. The trend toward lower corepressor expression in tamoxifen resistant tumors was exciting but not quite statistically significant, due to the cohort size in this study. However, a statistical probability table for each transcript suggests that a decrease in N-CoR may be predictive of tamoxifen resistance.

c. Quantitation of L7/SPA expression in expanded tumor group with known receptor status, for analysis of correlation to ER and PR.
A correlation had been observed in the initial group of ten tumors, between L7/SPA transcript expression and receptor status. Specifically, tumors that were ER+/PR+ expressed higher levels of L7/SPA than ER+/PR- or ER-/PR- tumors, suggesting that the transcript may be hormone responsive. When the preliminary findings were combined with the quantitations in the larger tumor cohort, a positive correlation was seen between L7/SPA levels and PR level, but not ER (**Fig.4**). This was seen when comparing receptor status (using 15 fmol/mg receptor as a +/- cutoff) and when correlating to absolute receptors levels (p=0.0071).

Task 3: Functional analysis of coregulator isoforms. Months 18-36.

a. Transfection studies of SMRT corepressor isoforms to measure suppression of partial agonist effects.
Both N-CoR and SMRT suppress the agonist activity of tamoxifen on an estrogen responsive reporter. This was demonstrated using the alternatively spliced Δ1330-1375 form of SMRT, which was the first SMRT construct cloned. However, full-length SMRT or the Δ1300-1375 isoform had not been fully characterized. As shown in **Fig.5**, when cloned into the same vector background, all three isoforms effectively suppressed the transcriptional activity of a tamoxifen-ER complex on an estrogen responsive reporter (pA3-ERE_2-$TATA_{tk}$-Luciferase). The effect was dose dependent. All three SMRT isoforms were maximally effective at 2μg DNA per 10 cm dish, suppressing the agonist activity of tamoxifen by 85 to 90% compared to controls lacking SMRT. Levels of transfected SMRT protein expression were not regulated by tamoxifen. The effectiveness of Δ1300-1375 SMRT was surprising, since this construct lacks most of the second RID of SMRT (characterized for retinoid and thyroid receptors, **Fig.2A**). RID-1 is also not required for the interaction between SMRT and tamoxifen-bound ER, since deletion of RID-1 from the full length or Δ1300-1375 SMRT did not abolish their function (**Fig.5**). Furthermore, an N-terminal SMRT protein lacking both RIDs (N-SMRT) was also able to suppress the activity of tamoxifen (**Fig.5**). Thus, tamoxifen-occupied ER must interact with SMRT at different RIDs than the ones currently mapped -- RID-2 which binds TR preferentially, or RID-1 which binds RAR.

b. Preparation and analysis of dominant negative SMRT and N-CoR expression constructs.
In studies utilizing C-terminal RID fragments of N-CoR and SMRT lacking repression domains, it has been shown by others that the repression activity of the full length N-CoR and SMRT

proteins could be abbrogated. This was done using retinoid and thyroid receptors, and the dominant negative activities of the C-terminal constructs were presumably due to their ability to bind receptors without repressing transcription, and to block binding of the functional corepressors. Given that we have seen that deletion of one or both of these characterized RIDs does not abolish the repressor activity of SMRT on tamoxifen-occupied ER (**Fig.5**), it is unlikely that these regions will act as dominant negative corepressors in this experimental model. Initial cotransfection experiments have confirmed this, and therefore this strategy may require replanning, and further charactization of the functional domains of the two corepressors.

c. Studies of SMRT and N-CoR suppression of coactivator enhanced partial agonist activity.

and

d. Hormone treatment of cell lines and quantitation of coregulator transcripts and proteins, to determine hormonal regulation of coregulator expression.

These two final sub-tasks remain to be completed.

Key Research Accomplishments

- Establishment of a sensitive, quantitative assay to measure levels of mRNA transcripts in breast tumor specimens and in cultured cell lines: this is particularly important in tissue samples, where the amount of available sample is very limited.
- Measurement of SMRT, N-CoR, L7/SPA and SRC-1 transcript levels in cell lines and tumors and correlation to tamoxifen sensitivity.
- Characterization of a previously undescribed alternatively spliced form of the corepressor SMRT.
- Analysis of repression of tamoxifen agonist activity by SMRT and mapping of repression effects.
- The discovery that a decrease in N-CoR transcript levels may be predictive of the development of tamoxifen resistance in breast cancer.
- Identification of a positive correlation between PR expression and the coactivator L7/SPA.
- Presentation of the research findings at three international meetings (abstracts appended).
- Compilation of the data into two review articles (appended)

Reportable Outcomes

The results described above are being compiled into a manuscript for submission to Cancer Research. At the time of this submission a copy of the manuscript will also be submitted to the USAMRMC.

The work has been briefly discussed in two review articles:

Graham, JD, Bain, DL, Richer, JK, Jackson, TA, Tung, L, Horwitz, KB (2000) Thoughts on tamoxifen resistant breast cancer. Are coregulators the answer or just a red herring? Journal of Steroid Biochemistry and Molecular Biology, in press.

Graham, JD, Bain, DL, Richer, JK, Jackson, TA, Tung, L, Horwitz, KB (2000) Nuclear receptor conformation, coregulators and tamoxifen resistant breast cancer. Steroids, in press.

The results have also been presented at three international scientific conferences:

Graham, JD, Tung, L, Fuqua, SAW, Osborne, CK, Horwitz, KB (1999) Do transcriptional corepressors control tamoxifen resistance in breast cancer? Proceedings of the Endocrine Society 81st Annual Meeting, USA, 1999.

Graham, JD, Abel, MG, Jackson, TA, Gordon, DF, Wood, WM, Horwitz, KB (2000) Novel interactors mediating mixed antagonist action on estrogen and progesterone receptors in breast cancer. Proceedings of the Keystone Symposium: Nuclear Receptors 2000, USA, 2000.

Graham J D, Abel M G, Gordon D F, Wood W M and Horwitz K B (2000) Receptor interacting proteins and the function of progesterone and estrogen receptors in breast cancer. Proceedings of the International Congress on Endocrinology 2000, Sydney, Australia, 2000.

Conclusions

A sensitive, quantitative RT-PCR assay has been successfully developed and applied to the problem of tamoxifen resistance in breast cancer. Using the assay, expression levels of the corepressors N-CoR and SMRT, and the coactivators SRC-1 and L7/SPA, were measured in a group of tamoxifen sensitive and resistant tumors. N-CoR and SMRT levels were overall lower in tamoxifen resistant tumors, compared with the tamoxifen sensitive group. Expression levels of the coactivators did not differ among the tumors. Transcriptional studies of SMRT demonstrated that its ability to suppress the partial agonist activity of tamoxifen is not mediated through the previously defined receptor interaction domains of that protein, suggesting that corepressors suppress partial agonist activity by a novel mechanism. Statistical analysis of my results suggests that **decreased levels of corepressor expression in breast tumors may predict the development of tamoxifen resistance**. *What about the coactivators?* The coactivators SRC-1 and L7/SPA may not be involved in tamoxifen resistance. However, **other coactivators that are yet to be defined may play a role in the increased partial agonist activities of mixed antagonists**, and the eventual transition to a resistant phenotype. We believe that the presently known coregulators, which appear to be involved in the development of tamoxifen resistance, represent a small subgroup of proteins, many of which are still to be defined. Therefore, in future work I plan to identify novel receptor-interacting proteins that play a role in tamoxifen resistance in breast cancer. Defining these new factors in hormone resistance will be the key to understanding and predicting this problem in ER positive breast cancer.

Appendices

Abstracts:

Presented at **Proceedings of the Endocrine Society 81st Annual Meeting, USA, 1999.**

Do transcriptional corepressors control tamoxifen resistance in breast cancer?

J. Dinny Graham[1], Lin Tung[1], Suzanne A.W. Fuqua[2], C. Kent Osborne[2] and Kathryn B. Horwitz[1]. 1. Dept Medicine/Endocrinology, University of Colorado Health Sciences Center Denver, CO 80262. 2. Dept Medicine/Oncology, University of Texas Health Science Center, San Antonio, TX 78284.

Therapy with the antiestrogen tamoxifen is one of the most effective strategies for the treatment of estrogen receptor (ER) positive breast cancers. However, tumors inevitably become tamoxifen resistant, despite continued expression of ER in most cases. We have proposed that "resistance" is due to an increase in the agonist activity of tamoxifen, resulting in inappropriate stimulation of the tumor. Related to this, we have found that certain transcriptional coactivators enhance, while corepressors inhibit, the partial agonist activity of mixed antagonists like tamoxifen, or the antiprogestin RU486. We therefore postulated that levels of transcriptional coregulators in a tumor may be important determinants of tamoxifen-treatment outcome. We have developed a sensitive and quantitative RT-PCR assay to measure transcript expression of two coactivators -- L7/SPA and SRC-1, and two corepressors -- N-CoR and SMRT. The assay was used to measure coregulator expression in tumors from tamoxifen-sensitive or -resistant patients. We report data from 5 tamoxifen-responsive patients with no disease recurrence for 2 to 5 years after the end of treatment; and 18 tamoxifen-resistant patients whose disease progressed during tamoxifen, or recurred within 6 months of tamoxifen cessation. These initial patients demonstrate a clear trend towards decreased corepressor expression in the tamoxifen-resistant patients, with mean levels of both N-CoR and SMRT reduced by 33% compared to the tamoxifen sensitive group. Surprisingly, SMRT is variably expressed as three distinct transcripts in solid tumors and in breast cancer cell lines. These transcripts encode full length SMRT (1405 amino acids); a previously described splice variant (Δ1330-1375); and a novel Δ1300-1375 splice variant which lacks the second of two C-terminal receptor interacting domains (RID-2). Transcriptional studies show that all three isoforms suppress the agonist activity of tamoxifen on an estrogen-responsive reporter, suggesting that RID-2 is not required for repression. Interestingly, deletion of RID-1 from either full-length or Δ1300-1375 SMRT, also does not disrupt the tamoxifen repressor function, suggesting that RID-1 too, is not required for repression. Thus SMRT mediated transcriptional repression of antagonist-occupied steroid receptors occurs through different mechanisms than SMRT-mediated repression of unliganded thyroid/retinoic acid receptors. This supports our hypothesis that that repression of antagonist-occupied steroid receptors by transcriptional corepressors represents a beneficial pharmacological accident that can be expoited in the search for novel ligands.

Presented at **Proceedings of the Keystone Symposium: Nuclear Receptors 2000, USA, 2000.**

Novel Interactors Mediating Mixed Antagonist Action on Estrogen and Progesterone Receptors in Breast Cancer.

J. Dinny Graham, M. Greg Abel, Twila A. Jackson, David F. Gordon, William M. Wood and Kathryn B. Horwitz. Division of Endocrinology, University of Colorado Hlth Sc Ctr, B151, 4200 E. 9th Ave, Denver, Colorado 80262, USA.

The antiestrogen tamoxifen is one of the most effective treatments for estrogen receptor (ER) positive breast cancer. However, tumors inevitably develop resistance to the treatment, which we postulated is due to the emergence of inappropriate agonist-like effects of this mixed antagonist. We have shown that the balance of agonist and antagonist activities of mixed antagonists is influenced by the abundance of nuclear receptor coregulators. We demonstrated that the corepressors N-CoR and SMRT, suppress the partial agonist activities of tamoxifen and the mixed antiprogestin RU486 on ER and progesterone receptor (PR), respectively. Furthermore, a novel coactivator, L7/SPA, enhances partial agonist activity. These effects are mixed antagonist-specific, and are not observed with agonists or pure antagonists. In addition, we found that the expression levels of these coregulators may differ between tamoxifen sensitive and resistant breast tumors, suggesting that they may be determinants of tamoxifen responsiveness. We postulated that other novel factors may play a specific role in determining mixed antagonist effects in breast cancer. We have employed two different antagonist-specific screening strategies to identify proteins involved in tamoxifen and RU486 action. Conventional yeast 2-hybrid screening was performed in the presence of RU486, with the hinge and hormone binding domain of PR as bait. We have identified a novel protein of approximately 109 kDa, which interacts with PR only when liganded to RU486. The protein contains eight nuclear receptor (NR) binding LXXLL domains. Mutagenesis of one out of two NR boxes, contained in the original 2-hybrid clone, resulted in loss of PR interaction with that fragment. The protein also contains three putative tetratricopeptide repeat domains, which may be involved in nuclear targeting of RU486-liganded PR and act as a scaffold for assembly of PR into multiprotein complexes. Recent evidence suggests that mixed antagonist-specific interactions with ER and PR involve multiple contacts with both AF-1 and AF-2 of the intact receptors. To screen for such proteins we have used a Sos recruitment 2-hybrid screening strategy with a full length ER bait, in the presence of tamoxifen. A number of antagonist-specific ER interacting proteins have been isolated and will be described.

Presented at **Proceedings of the International Congress on Endocrinology 2000, Sydney, Australia, 2000.**

Receptor Interacting Proteins and the Function of Progesterone and Estrogen Receptors in Breast Cancer

Graham J D, Abel M G, Gordon D F, Wood W M and Horwitz K B
Division of Endocrinology, University of Colorado Hlth Sc Ctr, Denver, Colorado 80262, USA.

The nuclear receptors for estrogen and progesterone (ER and PR) are important therapeutic determinants in breast cancer. Tumors expressing both receptors are generally well differentiated, indolent, and likely to respond to treatment with the mixed antiestrogen, tamoxifen. However, responsive tumors inevitably become tamoxifen-resistant and progress, often in the face of continued ER expression. We postulated that this is due to an increase in the partial agonist activity of tamoxifen. To test this hypothesis we have been searching for novel proteins that interact with receptors and modify the activities of mixed antagonists like tamoxifen. Using mixed antagonist-biased interaction screening, we have identified proteins that interact with ER and PR, and regulate transcription. The corepressors N-CoR and SMRT suppress the partial agonist activities of tamoxifen and the antiprogestin RU486, whereas the coactivator L7/SPA enhances this activity, yet has no effect on pure agonists or antagonists. In tamoxifen-resistant tumors removed from patients, we see a trend towards decreased expression of corepressors. In the same screen we identified a cDNA fragment encoding a novel protein, that we have now cloned and fully sequenced. The 109 kD protein interacts best with unliganded and mixed antagonist-bound PR, and less well with agonist-bound PR. The 944 amino acid protein sequence contains four nuclear receptor interaction LXXLL motifs. Additionally, there are three tetratricopeptide repeat (TPR) motifs in the N-terminus, characteristic of chaperonin/immunophilin binding proteins. Indeed, hsp90 also interacts with the protein strongly in protein interaction experiments. When expressed as a green fluorescent fusion protein, it shows a punctate cytoplasmic localization, which persists in the presence of progestins. We are testing the hypothesis that this protein has a scaffolding function, and plays an integral role in the correct expression and folding of nascent receptors, and perhaps their subcellular localization.

Tables:

Table 1. Coregulator transcript expression levels in breast tumors and cell lines.
N-CoR, SMRT, SRC-1 and L7/SPA expression levels were estimated by quantitative RT-PCR in total RNA isolated from 9 cell lines and 10 breast tumors. Each data point is the result of extrapolation from 5 duplicate determinations. The results are representative of 3 (cell lines) or 2 (tumors) determinations. Expression levels are shown relative to total RNA, and compared to ER and PR status in the tumors and cell lines.

Patient/Cell line	ER/PR	N-CoR (fmol/mg)	SMRT (fmol/mg)	L7/SPA (fmol/μg)	SRC-1 (fmol/mg)
1	+/+	0.903	0.586	2.715	0.156
2	+/+	0.888	0.417	2.437	0.048
3	+/+	2.390	0.132	5.372	0.094
4	-/+	0.236	0.993	0.331	0.564
5	-/+	0.602	0.490	0.561	0.199
6	-/-	0.000	0.055	0.000	0.044
7	-/-	1.659	0.235	0.511	0.258
8	-/-	1.627	0.424	0.262	0.179
9	-/-	0.473	0.999	0.429	0.147
10	-/-	0.000	0.331	0.315	0.148
T47Dco	-/+	3.186	2.619	26.480	0.694
T47D-Y	-/-	1.931	2.415	24.630	0.833
ZR-75-1	-/+	3.962	9.946	16.060	0.855
MCF-7	+/-	6.922	4.259	24.570	0.754
MDA-MB-231	-/-	10.832	6.910	36.260	0.419
HBL-100	-/-	6.842	6.378	4.210	0.603
HMEC	-/-	2.506	1.792	1.647	0.357
184	-/-	6.174	1.602	2.760	0.409
HeLa	-/-	10.347	3.940	54.040	0.268

Figures:

Figure 1: Detection and quantitation of coregulator transcripts in breast tumor total RNA. Endogenous transcripts in total tumor RNA were reverse transcribed and 20ng/reaction tube were PCR amplified in the presence of increasing amounts, in duplicate, of control template as indicated. PCR products were digested to cut the control, and visualized by Southern blotting. Products were quantified on a phosphorimager and endogenous to control N-CoR, SMRT, SRC-1 and L7/SPA levels were estimated in order to determine the levels of endogenous transcripts in the original tumor sample. In the example shown here, all four coregulators were visualized from the same ER+/PR+ tumor sample. The levels of each transcript were N-CoR: 1.448 fmol/mg, SMRT: 0.806 fmol/mg, SRC-1: 0.406 fmol/mg, L7/SPA: 2.122 fmol/μg total RNA.

Figure 2: Characterization of variant SMRT forms.
A. Organization of the SMRT full length protein, the previously described Δ1330-1375 splice variant, and a new Δ1300-1375 splice variant. Amino acid positions of defined repression and receptor interaction domains are indicated. SRD, SMRT repression domain. RID, receptor interaction domain. Shaded regions of RID-1 and RID-2 represent previously described glutamine-rich and α-helical regions, respectively. **B.** A representative southern blot of SMRT quantitative RT-PCR products amplified from the total RNA of MCF-7 breast cancer cells. Three SMRT transcripts were coamplified from 20ng total cellular RNA and 1 to 20 fg of the control template, present in duplicate. Full length SMRT and the Δ1330-1375 and Δ1300-1375 splice variants are indicated. **C.** Quantitation of the three SMRT isoforms in cell lines. The levels of three SMRT transcripts in the indicated cell lines were determined using quantitative RT-PCR. Each data point was extrapolated from 5 duplicate determinations.

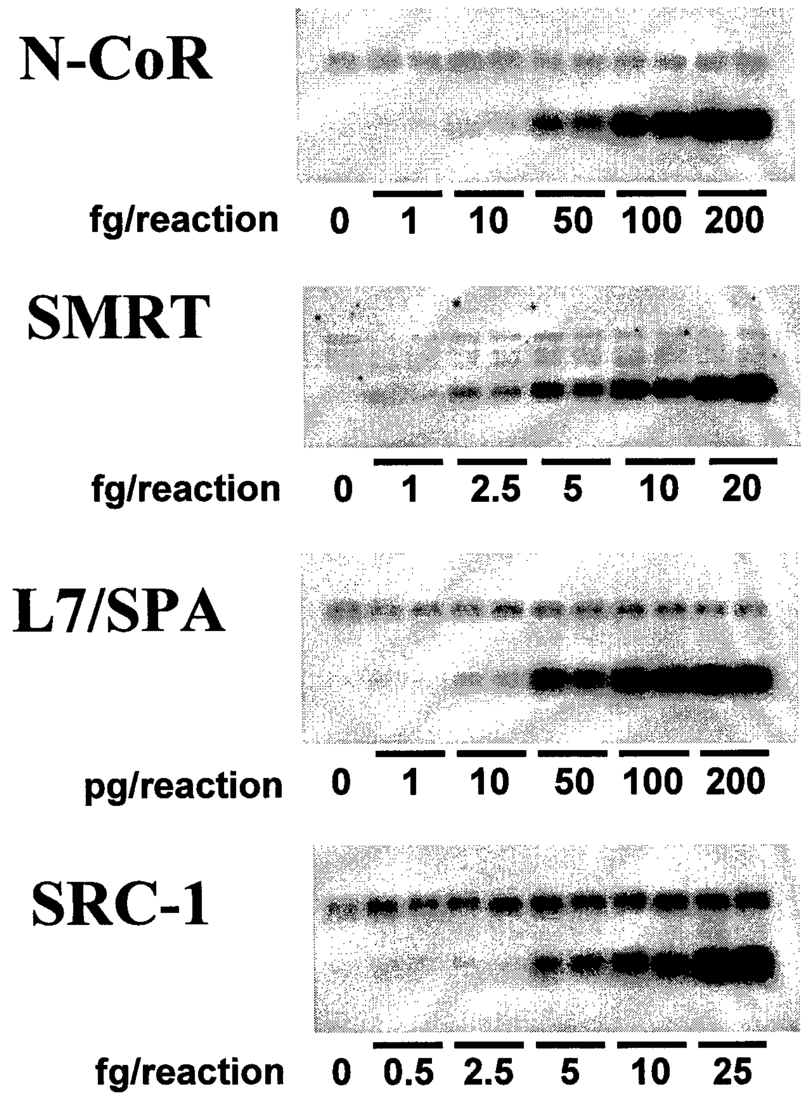
Figure 3: Coregulator expression in tamoxifen sensitive and resistant tumors.
Transcripts encoding the corepressors, N-CoR and SMRT, and the coactivators L7/SPA and SRC-1 were quantitated by RT-PCR in a cohort of 18 tamoxifen resistant and 6 sensitive breast tumors. The results are shown here as a box plot of sensitive vs. resistant tumor determinations and are based on the average of duplicate determinations per tumor. Boxes show the range of values between the 25th and 75th quartiles with median values shown as horizontal lines. There was a trend towards decreased corepressor levels in tamoxifen resistant patients. In particular, decreased N-CoR may be predictive for acquired tamoxifen resistance.

Figure 4: Correlation between L7/SPA and receptor status.
Levels of L7/SPA were compared to PR (panel A) and ER (panel B) status in tumor samples for which receptor status was available. Absolute L7/SPA levels are shown against receptor positivity, with a cut-off of 15fmol/mg by ER and PR enzyme immunoassay (EIA). L7/SPA expression was positively correlated with absolute PR level by EIA (p value = 0.0071). No correlation was seen between L7/SPA level and ER.

Figure 5: Deletion analysis of SMRT repression of tamoxifen partial agonist activity.
A. SMRT variant and deletion constructs used in transcriptional studies. Amino acid positions of defined repression and receptor interaction domains are indicated. SRD, SMRT repression domain. RID, receptor interaction domain. **B.** Transcriptional repression by SMRT constructs, of tamoxifen partial agonist activity. HeLa cells growing in phenol red-free MEM, supplemented

with 5% charcoal stripped FBS, were transfected with expression vectors for the three SMRT isoforms, full length or Δ1300-1375 SMRT lacking RID-1, or the N-SMRT construct lacking both RID-1 and RID-2 at 2μg/10cm dish, in the presence of 100ng of ER expression vector and 2μg ERE_2-$TATA_{tk}$-LUC reporter. Precipitates were removed 16 to 24h after transfection and cells were treated with 100nM tamoxifen or vehicle for 24h thereafter. Relative luciferase activities induced by tamoxifen in SMRT-overexpressing cells, are shown as a percentage of the tamoxifen-treated control. The data are representative of four experiments where the same results were seen.

Figure 1



N-CoR
fg/reaction 0 1 10 50 100 200
SMRT
fg/reaction 0 1 2.5 5 10 20
L7/SPA
pg/reaction 0 1 10 50 100 200
SRC-1
fg/reaction 0 0.5 2.5 5 10 25

Figure 2

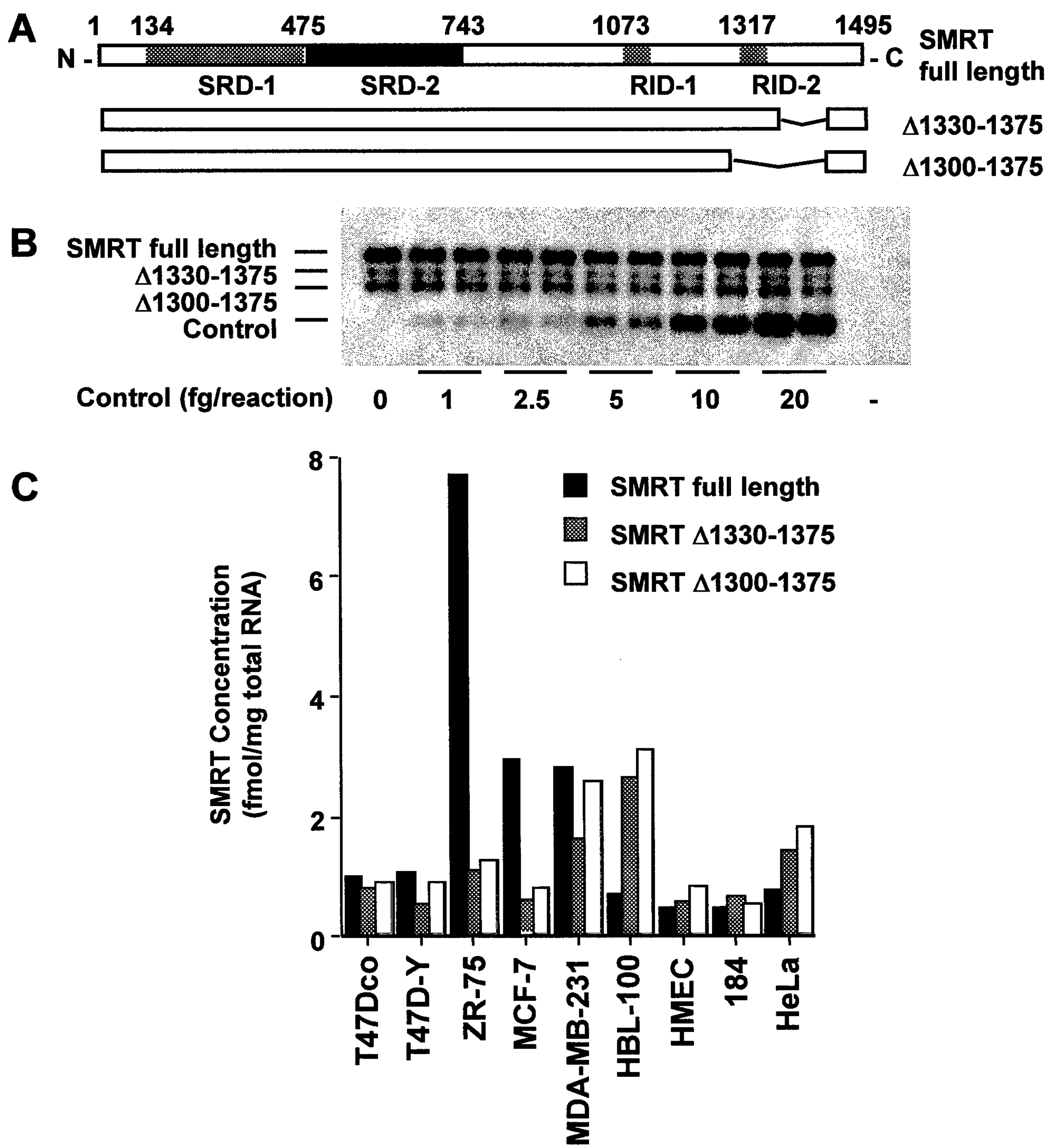

A
1
134
475
743
1073
1317
1495
N -
- C
SMRT full length
SRD-1
SRD-2
RID-1
RID-2
Δ1330-1375
Δ1300-1375
B
SMRT full length
Δ1330-1375
Δ1300-1375
Control
Control (fg/reaction)
0
1
2.5
5
10
20
-
C
SMRT Concentration (fmol/mg total RNA)
0
2
4
6
8
SMRT full length
SMRT Δ1330-1375
SMRT Δ1300-1375
T47Dco
T47D-Y
ZR-75
MCF-7
MDA-MB-231
HBL-100
HMEC
184
HeLa

Figure 3

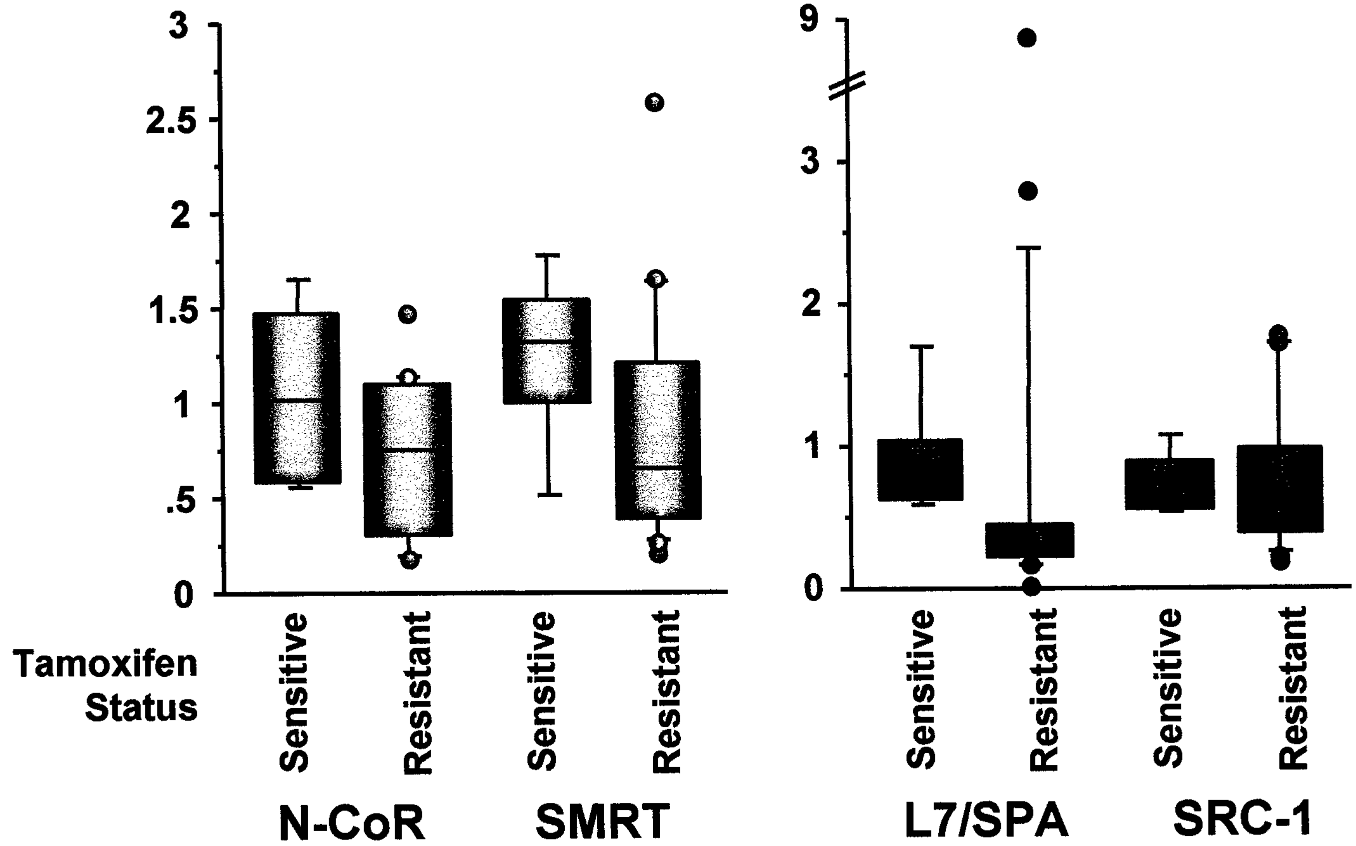

3
2.5
2
1.5
1
.5
0
Tamoxifen Status
Sensitive
Resistant
Sensitive
Resistant
N-CoR
SMRT
9
3
2
1
0
Sensitive
Resistant
Sensitive
Resistant
L7/SPA
SRC-1

Figure 4

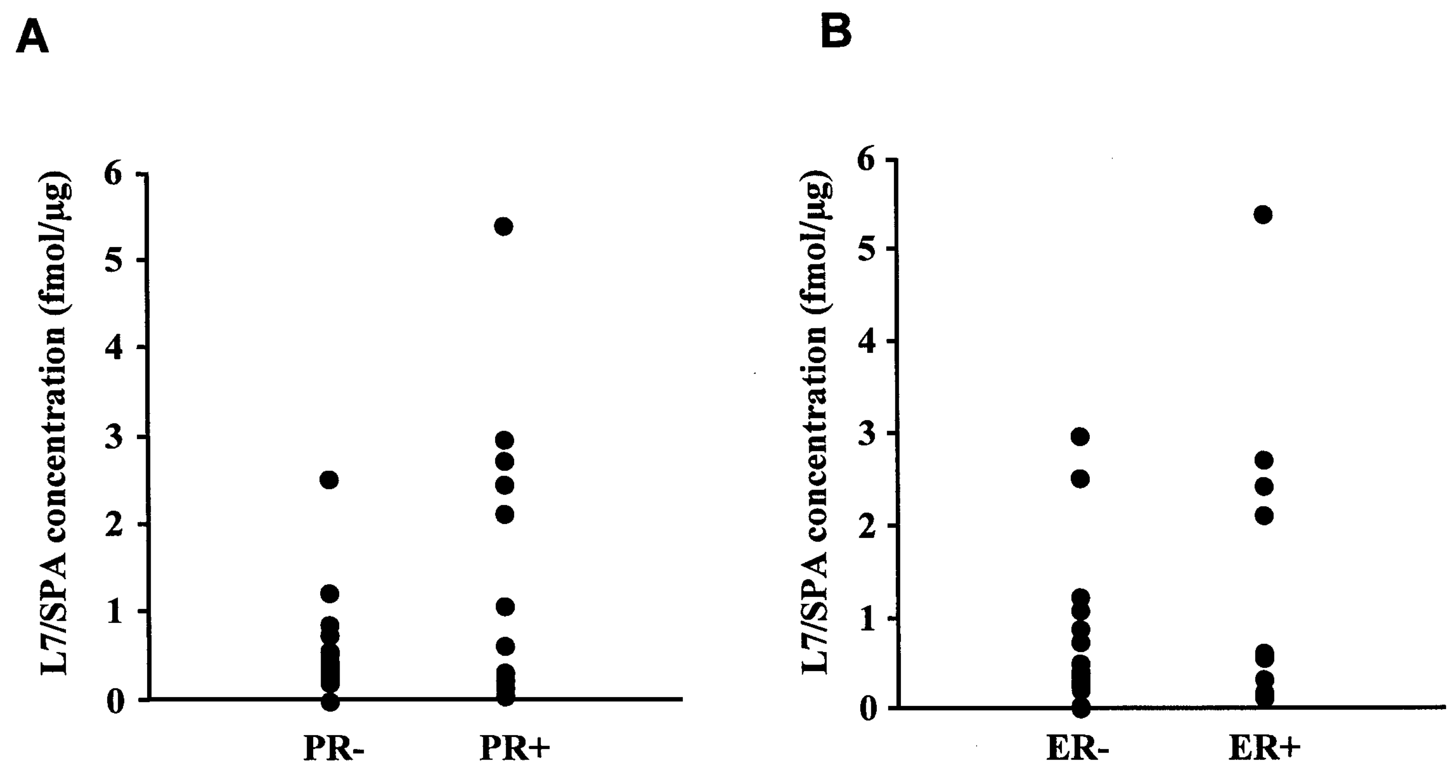
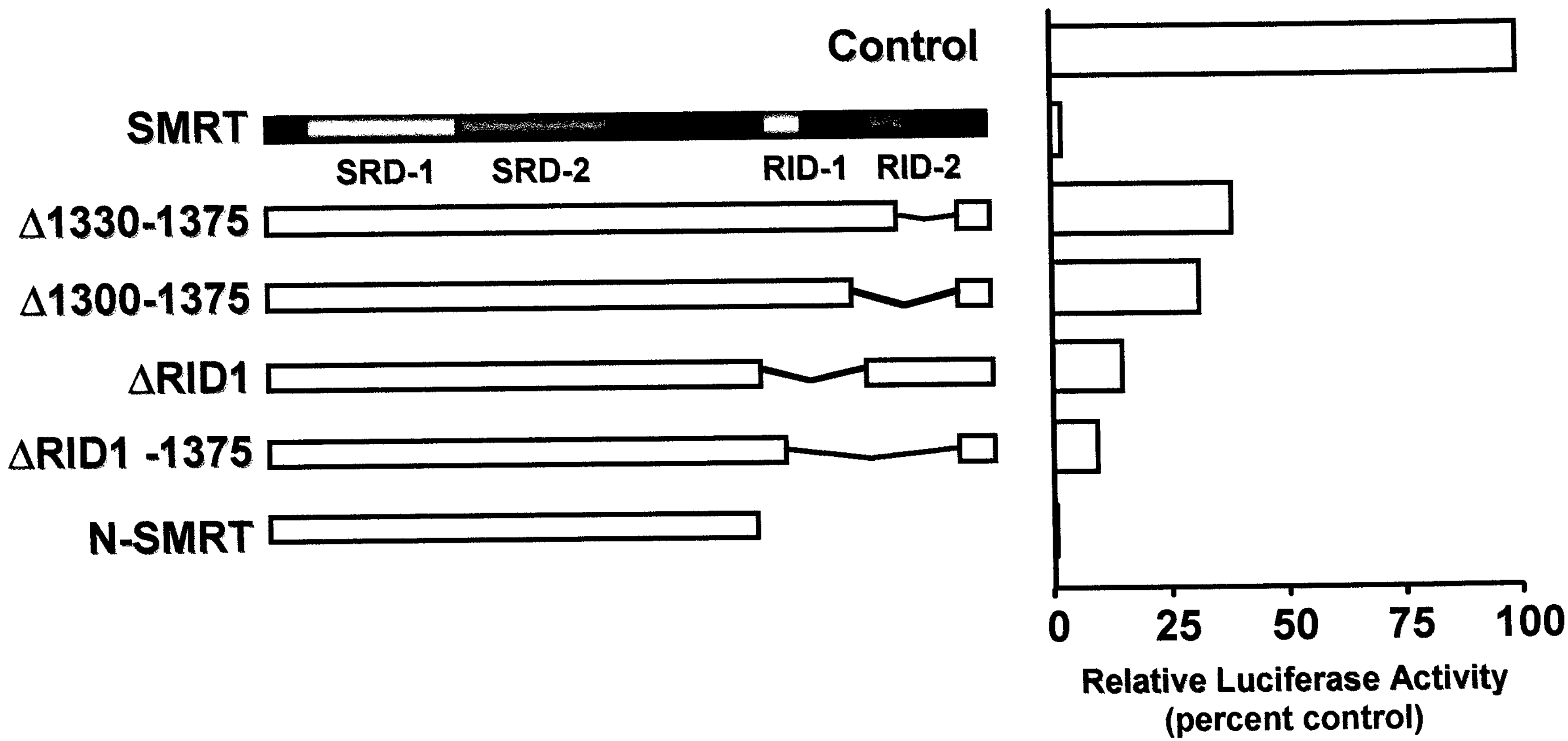


Figure 5



Control
SMRT
SRD-1
SRD-2
RID-1
RID-2
Δ1330-1375
Δ1300-1375
ΔRID1
ΔRID1 -1375
N-SMRT
0
25
50
75
100
Relative Luciferase Activity
(percent control)

Manuscripts:

Page proofs of the following manuscripts are appended:

Graham, JD, Bain, DL, Richer, JK, Jackson, TA, Tung, L, Horwitz, KB (2000) Thoughts on tamoxifen resistant breast cancer. Are coregulators the answer or just a red herring? Journal of Steroid Biochemistry and Molecular Biology, in press.

Graham, JD, Bain, DL, Richer, JK, Jackson, TA, Tung, L, Horwitz, KB (2000) Nuclear receptor conformation, coregulators and tamoxifen resistant breast cancer. Steroids, in press.

PERGAMON

Journal of Steroid Biochemistry & Molecular Biology 000 (2000) 000–000

The Journal of Steroid Biochemistry & Molecular Biology

www.elsevier.com/locate/jsbmb

Thoughts on tamoxifen resistant breast cancer. Are coregulators the answer or just a red herring?

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The antiestrogen tamoxifen is the most commonly used and effective treatment for patients with estrogen receptor (ER)-positive breast cancers. As an adjuvant for primary breast cancer, treatment with tamoxifen improves disease-free and overall survival rates [1] and in metastatic disease tamoxifen induces remission of ER-positive tumors [2,3]. Most recently, tamoxifen has been shown to prevent breast cancers in women at high risk of developing this disease [4]. Thus in various settings, tamoxifen is an inhibitory ER ligand in the breast, and this property explains both its efficacy and its widespread use.

However, almost without exception, breast cancers that initially respond well to tamoxifen by growth cessation or regression, eventually resume growing despite continued presence of the antagonist. How can this 'acquired resistance' be explained? It is likely, that in some cases, loss of ER expression or increased metabolism of the drug [5–7] accounts for loss of tumor responsiveness to tamoxifen. However, in more than half of acquired resistance cases, tumor ER are retained [8] — a statistic analogous to that seen with sequential ER measurements in relapsing patients who did not receive endocrine therapy [9]. Thus, tamoxifen treatment itself, does not significantly alter ER levels [9–11]. Indeed, tamoxifen-resistant tumors remain responsive to growth inhibition by pure antiestrogens (but clinical data are sparse) and other hormonal therapies [12]. Paradoxic reports of tumor stasis and even regression after tamoxifen withdrawal in resistant patients suggest that in at least some resistant tumors, the antagonist has switched to an agonist [13–15]. This is not entirely surprising, since tamoxifen is a 'mixed' antiestrogen, having primarily antagonist effects in the normal breast, but acting as an agonist in normal uterus and bone. These tissue-specific properties also classify tamoxifen as a 'selective estrogen receptor modulator' or SERM. We and others have suggested that the intrinsic estrogenic activity of tamoxifen observed in some normal tissues, may also account for acquired resistance in some breast cancers, resulting in inappropriate stimulation of tumors [reviewed in [16–19]]. New molecular studies of steroid receptors provide insights into mechanisms by which this may occur. However, they raise other questions to which we do not yet have answers.

ER, the direct targets of tamoxifen, are members of the nuclear receptor family of ligand activated transcription factors. In response to ligand, ER dimerize and bind to DNA response elements on the promoters of target genes to regulate transcription [20]. The extent and direction of gene regulation by ER is influenced not only by the types of ligands bound, but also by specific coregulatory proteins, present at rate-limiting levels in the nucleus, that are recruited to, and interact with promoter-bound receptor-ligand complexes [21–23]. These coregulatory proteins can be either coactivators, which enhance transcription, or corepressors which suppress it.

A number of coactivators are known to interact with agonist-occupied ER to enhance transcription. These include the p160 family of related coactivators [SRC-1, GRIP1/TIF2 and AIB1/RAC3/ACTR/p/CIP, refs [24–27]], CREB-binding protein CBP/p300 [28], and p/CAF, the CBP/p300-associated factor. These proteins form multiple contacts with ER and each other, to produce multi-protein cooperative coactivator complexes capable of synergistically activating estrogen-driven transcription [reviewed in [22,26,28,29]]. The complex has at least dual functions; it possesses histone acetyl-transferase activity [30–32], which facilitates chromatin remodeling, and it binds proteins of the

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PII: S0960-0760(00)00101-1

basal transcriptional machinery [33]. Like estradiol, the partial agonist transcriptional activity of tamoxifen appears also to be enhanced by coactivators. These include, in addition to p160 family members [34], a novel coactivator termed L7/Switch Protein for Anatagonists (L7/SPA; [35]. Unlike the p160 family of proteins, the action of L7/SPA is specific for mixed antagonists; it does not enhance the activity of estradiol-bound ER or progesterone-bound progesterone receptors (PR).

Two nuclear receptor corepressors have been identified to date: N-CoR (nuclear receptor corepressor) [36] and SMRT (silencing mediator for retinoid and thyroid receptors)/TRAC-2 [37,38]. Both were initially characterized on the basis of their ability to bind members of the thyroid and retinoid receptor family of nuclear receptors in the absence of ligand, and repress transcription. In addition to associating with these nuclear receptors on DNA, N-CoR and SMRT form complexes with the mammalian repressor mSin3 and with histone deacetylases [[39–41], and reviewed in [42]], which together repress chromatin structure and block transcription. Unlike the unliganded thyroid/retinoic acid family of nuclear receptors, neither unliganded steroid receptors, nor agonist-bound ER and PR bind N-CoR or SMRT. Thus, under physiological circumstances, there are no known associations between steroid receptors and transcriptional corepressors.

To test the hypothesis that mixed antagonists, like tamoxifen or the antiprogestin RU486, have inappropriate agonist-like effects in certain tissues or tumors, we postulated that transcriptional coregulators are inadvertently brought to the promoters of DNA-bound, antagonist-occupied receptors, and set out to isolate such factors. Initially, the PR C-terminus, consisting of the hinge and hormone binding domain of the receptors was used as bait in a two-hybrid screen of a HeLa cDNA library, in which the yeast cells were treated with saturating concentrations of RU486 [35]. Thus we biased the system in an attempt to isolate factors that preferentially interact with antagonist-occupied receptors. We isolated several interesting proteins that regulate transcription by antagonist-occupied receptors in opposite directions. One was L7/SPA, a previously described 27 kD protein containing a basic region leucine zipper domain at its N-terminus, through which it forms stable homodimers that bind to RNA and double-stranded DNA (reviewed in Jackson et al., 1997). A Green-Fluorescent-Protein-L7/SPA chimera localizes to the nucleus. When coexpressed with tamoxifen-occupied ER, or RU486-occupied PR or glucocorticoid receptors (GR), L7/SPA increases the partial agonist activity of the antagonists by 3 to 10-fold. Importantly, it has no effect on agonist-mediated transcription involving estradiol, progesterone or dexamethasone. The interaction of L7/SPA with PR maps to the hinge region of the receptors, and indeed, the isolated PR hinge region squelches, or inhibits, L7/SPA-dependent increases in transcription by tamoxifen [35]. This protein does not bind to the hinge region via the LXXLL motifs characteristic of coactivators that bind at activation function 2 of the hormone binding domain. Interestingly, transcription by pure antagonists which lack partial agonist actions, such as the pure antiestrogen ICI164,384 or the pure antiprogestin ZK98299, can not be upregulated by L7/SPA. Therefore, this coactivator appears to up-regulate specifically, that component of transcription activatable by mixed antagonists [35].

In the same protein–protein interaction screen in which L7/SPA was isolated, we also isolated a C-terminal human (h) cDNA fragment that turned out to be homologous to the mouse (m) N-CoR whose sequence had been published several weeks earlier [37]. We cloned and sequenced the entire human N-CoR coding sequence, and compared it with the murine coding sequence. In addition to a 7359 nucleotide open reading frame that predicts a 2453 amino acid protein, two apparent N-terminal splice variants that result in loss of amino acids 83-206 and amino acids 83-147 in the N-terminal repressor domain, were detected in the human transcripts. The amino acid identity between mN-CoR and hN-CoR is high (98.9%), with the greatest divergence observed in the second repressor domain, in which the identity falls to 80.4%. Binding of hN-CoR maps to the PR hormone binding domain. We found that mN-CoR, and the related human corepressor, SMRT suppress the partial agonist activity of RU486 or tamoxifen by more than 90%. This suppression is completely squelched by overexpression of the transcriptionally silent PR C-terminus. These studies represented the first demonstration that antagonist-occupied steroid receptors can recruit corepressors to the transcription complex [35]. They suggest that the effects of antagonist ligands are not passive — i.e. that they do not simply prevent transcription by blocking binding and activation of receptors by agonists. Instead, these studies suggest that repression by antagonists can be an active process, generated via recruitment of corepressor molecules to the transcriptional machinery, by antagonist-receptor complexes. In this scenario, steroid antagonists can theoretically suppress transcription even in the absence of the cognate agonists. For example, antagonists may suppress transcription of a gene whose promoter contains a steroid response element, but which is activated by growth factor signaling pathways that regulate the same promoter. This would explain observations of breast tumor growth suppression by antagonists, which occur even in the absence of the agonist.

Additional studies demonstrated that corepressors can reverse the transcriptional activation produced by antagonist ligands in the presence of the coactivator, L7/SPA [35]. This suggested to us, that the relative levels of coactivators vs corepressors may determine whether the agonist or antagonist effects of these mixed antagonists predominate in a tissue or tumor. Although there is clearly functional redundancy among coregulatory proteins, there is evidence to suggest that their expression levels are rate-limiting, and therefore, that their relative levels may determine the outcome of ligand signaling in a cell. Specifically, with respect to tamoxifen resistant breast cancers, we hypothesized that the levels of expression of coactivators versus corepressors in a tumor would dictate whether tamoxifen exerts agonist- or antagonist-like activity. For example, under conditions of coactivator excess, in which the agonist-like activity predominates, the tumor would respond inappropriately to tamoxifen. Such a tumor would present with a 'resistant' phenotype, yet would actually be responding to tamoxifen, albeit as an agonist. To test this idea we have begun measuring transcript expression levels of several coregulators in breast tumors known to be tamoxifen sensitive or resistant. We have developed a sensitive, quantitative reverse transcription–polymerase chain reaction (RT-PCR) protocol to measure mRNA levels encoding the corepressors N-CoR and SMRT and the coactivators L7/SPA and SRC-1. This assay accurately measures the extremely low levels of these factors present in small amounts of tumor material in a set of tumors in which the tamoxifen-response status has been defined. The studies are ongoing, but our preliminary data indicate that corepressor levels may be more important than coactivator levels. Additionally, the studies indicate that mechanisms of corepressor binding to steroid receptors may differ from their binding to thyroid/retinoic acid receptors. This could have been predicted, given the fact that steroid receptors are not natural targets of corepressor activity. Rather, their recruitment to antagonist-occupied steroid receptors may represent a pharmacologic anomaly [43].

It is our opinion, that the full complement of coregulatory proteins that could influence the direction of transcription by antagonist-occupied steroid receptors has yet to be completely identified. L7/SPA is the only antagonist-specific coactivator defined to date. We doubt that it is the only, or even the most important, protein to have this property. Similarly, it is unlikely that SMRT and N-CoR are the only corepressors that interact with antagonist-occupied steroid receptors. We believe that these three coregulators represent a minor subset of the whole, for several reasons. First, screening for coregulators has, for the most part, been limited to proteins that interact with the C-terminal ligand binding domain of the receptors at activation function 2. However, activation function 1, located in the N-terminus of the receptors, may be as important, if not more important for the agonist properties of steroid antagonists, but this region has received little attention as a target for coregulatory protein binding. Newer experimental strategies may correct this deficiency [44,45]. Second, recent crystallographic analyses of steroid receptor ligand binding domains show that there are subtle structural variations in the conformation of receptors resulting from the binding of different ligands [46,47]. This would yield multiple, subtly different targets on the C-terminal surface of the receptors, for the binding of a variety of coregulators, dictated specifically by the identity of the ligand. As discussed above, few, if any, ligand-specific coregulators have yet been identified, perhaps because multiple ligands have not been compared in screening assays. Third, while C-terminal receptor structure has been analyzed in detail, little is known about the structure of steroid receptor N-termini. Yet, to judge from PR [48], this region has an ordered but asymmetric structure, that would offer multiple potential target sites for protein–protein interactions. The structure of steroid receptor N-termini appears to be influenced by the DNA binding domain, and possibly, also by DNA binding-induced allostery. Putative recruitment of coregulatory proteins could therefore occur either through induced fold mechanisms generated by direct contact between the coregulators and the receptors at various sites along the N-terminus, or through structural alterations in the N-terminus (followed by binding of different coregulators) induced by DNA binding. The latter in particular, suggests a scenario in which different coregulators interact with receptors, depending on the gene to which the receptors are bound. Existence of such heterogeneity is suggested by the known tissue specificity of antagonists. For example, the usefulness of tamoxifen in breast cancer is due to its predominantly antagonist nature in the breast. However, in the uterus it is a potent estrogen, where, like estradiol (when unopposed by progesterone) it can induce epithelial hyperplasia and endometrial cancers. Are tamoxifen's mixed agonist/antagonist properties due to (i) activation of different sets of genes in the breast vs uterus; (ii) the presence of different sets of coregulators in each tissue; (iii) varying and limiting levels of coregulator subsets in each tissue; or (iv) differential utilization of a common set of coregulators whose recruitment to the receptors is controlled by the identity of the ligand and by variable DNA structure at different gene loci?

The answers to these questions have important implications for our understanding of acquired tamoxifen resistance in breast cancer. Mechanism (i) above is likely to be the case, as gene array technology is beginning to show. On the other hand, to date, no tissue-specific coregulators have been described. Quite the contrary, all known coregulators are 'ubiquitous'. This suggests that mechanism (ii) may not be critically important, and if anything, that number (iii), which addresses the relative levels of coregulators, is more important. However, this scenario does not address the redundancy issue. Namely, the possibility that one coregulator can functionally replace another. Coregulator redundancy is suggested by SRC-1 knockout studies, which generated only subtle phenotypes in the SRC-1 deficient mice [49]. If the same coregulator complement and redundancy exists in breast cancers, it will, we believe, make it very difficult to correlate tamoxifen resistance with coregulator levels. That leaves scenario number (iv) above as a likely player in tamoxifen resistance — an extraordinarily complex one.

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Steroids 65 (2000) 000–000

Nuclear receptor conformation, coregulators, and tamoxifen-resistant breast cancer

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Abstract

The development of tamoxifen resistance and consequent disease progression are common occurrences in breast cancers, often despite the continuing expression of estrogen receptors (ER). Tamoxifen is a mixed antagonist, having both agonist and antagonist properties. We have suggested that the development of tamoxifen resistance is associated with an increase in its agonist-like properties, resulting in loss of antagonist effects or even inappropriate tumor stimulation. Nuclear receptor function is influenced by a family of transcriptional coregulators, that either enhance or suppress transcriptional activity. Using a mixed antagonist-biased two-hybrid screening strategy, we identified two such proteins: the human homolog of the nuclear receptor corepressor, N-CoR, and a novel coactivator, L7/SPA (Switch Protein for Antagonists). In transcriptional studies, N-CoR suppressed the agonist properties of tamoxifen and RU486, and L7/SPA increased agonist effects. We speculated that the relative levels of these coactivators and corepressors may determine the balance of agonist and antagonist properties of mixed antagonists, such as tamoxifen. Using quantitative RT-PCR, we, therefore, measured the levels of transcripts encoding these coregulators, as well as the corepressor SMRT, and the coactivator SRC-1, in a small cohort of tamoxifen-resistant and sensitive breast tumors. The results suggest that tumor sensitivity to mixed antagonists may be governed by a complex set of transcription factors, which we are only now beginning to understand.

Keywords: Steroid receptors; Estrogen receptors; Progesterone; Breast neoplasms; Transcriptional coregulators; Tamoxifen

1. Introduction

For more than two decades, the antiestrogen tamoxifen has served as an effective treatment for estrogen receptor (ER)-positive breast cancers. First demonstrated to induce remission of advanced, metastatic disease [1,2], and later used as an adjuvant for ER-positive primary breast cancer, tamoxifen improves disease-free and overall survival [3]. Most recently, tamoxifen has been shown to reduce the likelihood of breast cancer in women at high risk of developing this disease [4]. Thus, in various settings, tamoxifen is an inhibitory ER ligand in the breast, and this property explains both its efficacy and its widespread use.

However, almost without exception, breast cancers that initially respond well to tamoxifen by growth cessation or regression eventually resume growing despite continued presence of the antagonist. How can this "acquired resistance" be explained? It is likely that, in some cases, loss of ER expression or increased metabolism of the drug [5–7] accounts for loss of tumor responsiveness to tamoxifen. However, in more than half of acquired resistance cases, tumor ER are retained [8]—a statistic analogous to that seen with sequential ER measurements in relapsing patients who did not receive endocrine therapy [9]. Thus, tamoxifen treatment itself does not significantly alter ER levels [9–11]. Indeed, tamoxifen-resistant tumors remain responsive to growth inhibition by pure antiestrogens (but clinical data are sparse) and other hormonal therapies [12]. Paradoxic reports of tumor stasis and even regression after tamoxifen withdrawal in occasional patients also suggest that, in at least some resistant tumors, the antagonist has switched to an agonist [13–15]. This is not entirely surprising since tamoxifen is a "mixed" antiestrogen, having primarily antagonist effects in the normal breast but acting as an agonist in normal uterus and bone. These tissue-specific properties also classify tamoxifen as a "selective estrogen receptor modulator" or SERM. We and others have suggested that the intrinsic estrogenic activity of tamoxifen observed in

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0039-128X/00/$ – see front matter
PII: S0039-128X(00)00116-1

some normal tissues may also account for acquired resistance in some breast cancers, resulting in inappropriate stimulation of tumors (reviewed in refs. 16–19). New molecular studies of steroid receptors provide insights into mechanisms by which this resistance may occur. However, these studies raise other questions to which we do not yet have answers.

ERs, the direct targets of tamoxifen, are members of the nuclear receptor family of ligand-activated transcription factors. In response to ligand, ERs dimerize and bind to DNA response elements on the promoters of target genes to regulate transcription [20]. The extent and direction of gene regulation by ERs are influenced not only by the types of ligands bound but also by specific coregulatory proteins, present at rate-limiting levels in the nucleus that are recruited to, and interact with, promoter-bound receptor–ligand complexes [21–23]. These coregulatory proteins can be either coactivators, which enhance transcription, or corepressors, which suppress it.

A number of coactivators are known to interact with agonist-occupied ER to enhance transcription. These include the p160 family of related coactivators: SRC-1, GRIP1/TIF2, and AIB1/RAC3/ACTR/p/CIP [24–27], CREB-binding protein CBP/p300 [28], and p/CAF, the CBP/p300-associated factor. These proteins form multiple contacts with ER and each other, to produce multi-protein cooperative coactivator complexes capable of synergistically activating estrogen-driven transcription (reviewed in refs. 22,26,28,29). The complex has at least dual functions: it possesses histone acetyl-transferase activity [30–32], which facilitates chromatin remodeling; and it binds proteins of the basal transcriptional machinery [33]. Like estradiol, the partial agonist transcriptional activity of tamoxifen appears also to be enhanced by coactivators. These include, in addition to p160 family members [34], a novel coactivator termed L7/Switch Protein for Anatagonists (L7/SPA) [35]. Unlike the p160 family of proteins, the action of L7/SPA is specific for mixed antagonists; it does not enhance the activity of estradiol-bound ER or progesterone-bound progesterone receptors (PRs).

Two nuclear receptor corepressors have been identified to date: N-CoR (nuclear receptor corepressor) [36] and SMRT (silencing mediator for retinoid and thyroid receptors)/TRAC-2 [37,38]. Both were initially characterized on the basis of their ability to bind members of the thyroid and retinoid receptor family of nuclear receptors in the absence of ligand, and repress transcription. In the presence of ligand, the corepressors dissociate, and transcription proceeds. In addition to associating with these nuclear receptors on DNA, N-CoR and SMRT form complexes with the mammalian repressor mSin3 and with histone deacetylases (39–41, and reviewed in ref. 42), which together repress chromatin structure and block transcription. Unlike the unliganded thyroid/retinoic acid family of nuclear receptors, neither unliganded steroid receptors nor agonist-bound ER and PR bind N-CoR or SMRT. Thus, under physiological circumstances, there are no known associations between steroid receptors and transcriptional corepressors. However, we speculated that the nonphysiological conformations induced by synthetic ligands may render the receptors target to such interactions.

2. Results

To test the hypothesis that mixed antagonists, like tamoxifen or the antiprogestin RU486, have inappropriate agonist-like effects in certain tissues or tumors, we postulated that transcriptional coregulators are inadvertently brought to the promoters of DNA-bound, antagonist-occupied receptors. We set out to isolate such factors. Initially, the PR C-terminus, consisting of the hinge and hormone-binding domain of the receptors, was used as bait in a two-hybrid screen of a HeLa cDNA library, in which the yeast cells were treated with saturating concentrations of RU486 [35]. Thus, we biased the system in an attempt to isolate factors that preferentially interact with antagonist-occupied receptors. We isolated several interesting proteins that regulate transcription by antagonist-occupied receptors in opposite directions. One was L7/SPA, a previously described 27-kDa protein containing a basic region leucine zipper domain at its N terminus, through which it forms stable homodimers that bind to RNA and double-stranded DNA (reviewed in ref. 35). A Green-Fluorescent-Protein-L7/SPA chimera localizes to the nucleus. When coexpressed with tamoxifen-occupied ER, or RU486-occupied PR or glucocorticoid receptors (GR), L7/SPA increases the partial agonist activity of the antagonists by 3- to 10-fold. Importantly, it has no effect on agonist-mediated transcription involving estradiol, progesterone, or dexamethasone. The interaction of L7/SPA with PR maps to the hinge region of the receptors, and indeed the isolated PR hinge region squelches, or inhibits, L7/SPA-dependent increases in transcription by tamoxifen [35]. This protein does not bind to the hinge region via the LXXLL motifs characteristic of coactivators that bind at activation function 2 of the hormone-binding domain. Interestingly, transcription by pure antagonists that lack partial agonist actions, such as the pure antiestrogen ICI164,384 or the pure antiprogestin ZK98299, can not be upregulated by L7/SPA. Therefore, this coactivator appears to up-regulate specifically that component of transcription activatable by mixed antagonists [35].

In the same protein–protein interaction screen in which L7/SPA was isolated, we also isolated a C-terminal human (h) cDNA fragment that turned out to be homologous to the mouse (m) N-CoR whose sequence had been recently published [37]. We cloned and sequenced the entire human N-CoR coding sequence and compared it with the murine coding sequence. In addition to a 7359-nucleotide open reading frame that predicts a 2453-amino acid protein, two apparent N-terminal splice variants that result in loss of amino acids 83–206 and amino acids 83–147 in the N-terminal repressor domain were detected in the human transcripts. The amino acid identity between mN-CoR and hN-CoR is high (98.9%), with the greatest divergence observed in the second repressor domain in which the identity falls to 80.4%. Binding of hN-CoR maps to the PR hormone binding domain. We found that mN-CoR and the related human corepressor, SMRT, suppress the partial agonist activity of RU486 or tamoxifen by more than 90%. This suppression is completely squelched by overexpression of the transcriptionally silent PR C terminus. These studies represented the first demonstration that antagonist-occupied steroid receptors can recruit corepressors to the transcription complex [35]. They suggest that the effects of antagonist ligands are not passive—i.e. that they do not simply prevent transcription by blocking binding and activation of receptors by agonists. Instead, these studies suggest that repression by antagonists can be an active process, generated via recruitment of corepressor molecules to the transcriptional machinery by antagonist-receptor complexes. In this scenario, steroid antagonists can theoretically suppress transcription even in the absence of the cognate agonists. For example, antagonists may suppress transcription of a gene whose promoter contains a steroid response element, but which is activated by growth factor signaling pathways that regulate the same promoter. This would explain observations of breast tumor growth suppression by antagonists, which occurs even in the absence of the agonist.

Additional studies demonstrated that corepressors can reverse the transcriptional activation produced by antagonist ligands in the presence of the coactivator, L7/SPA [35]. This suggested to us that the relative levels of coactivators versus corepressors may determine whether the agonist or antagonist effects of these mixed antagonists predominate in a tissue or tumor. Although there is clearly functional redundancy among coregulatory proteins, there is evidence to suggest that their expression levels are rate-limiting, and therefore that their relative levels may determine the outcome of ligand signaling in a cell. Specifically, with respect to tamoxifen-resistant breast cancers, we hypothesized that the levels of expression of coactivators versus corepressors in a tumor would dictate whether tamoxifen exerts agonist- or antagonist-like activity. For example, under conditions of coactivator excess, in which the agonist-like activity predominates, the tumor would respond inappropriately to tamoxifen. Such a tumor would present with a "resistant" phenotype, yet would actually be responding to tamoxifen, albeit as an agonist.

To test this idea, we have begun measuring transcript expression levels of several coregulators in breast tumors known to be tamoxifen-sensitive or resistant. We have developed a sensitive, quantitative reverse transcription-polymerase chain reaction (RT-PCR) protocol to measure mRNA levels encoding the corepressors N-CoR and SMRT and the coactivators L7/SPA and SRC-1. This assay accurately measures the extremely low levels of these factors present in small amounts of tumor material in a set of tumors in which the tamoxifen-response status has been defined. The studies are ongoing, but our preliminary data indicate that corepressor levels may be more important than coactivator levels (Fig. 1). Additionally, the studies indicate F1 that mechanisms of corepressor binding to steroid receptors may differ from their binding to thyroid/retinoic acid receptors. This could have been predicted, given the fact that steroid receptors are not natural targets of corepressor activity. Rather, their recruitment to antagonist-occupied steroid receptors may represent a pharmacologic anomaly [43]. A small study examining this same issue found little variation in the mRNA levels of coregulators TIF-1, SUG-1, RIP140, or SMRT among a group of 21 control, 6 short-term tamoxifen-treated, and 19 tamoxifen-resistant breast tumors. In fact, any slight differences in coregulator levels were in the opposite direction to those hypothesized [44]. However, in a nude mouse model, tumors derived from tamoxifen-resistant human breast cancer cells expressed lower levels of N-CoR protein than tamoxifen-sensitive controls [45], in concordance with our findings.

3. Discussion and comments

It is our opinion that the full complement of coregulatory proteins that could influence the direction of transcription by antagonist-occupied steroid receptors has yet to be completely identified. L7/SPA is the only antagonist-specific coactivator defined to date. We doubt that it is the only, or even the most important, protein to have this property. Similarly, it is unlikely that SMRT and N-CoR are the only corepressors that interact with antagonist-occupied steroid receptors. We believe that these three coregulators represent a minor subset of the whole, for several reasons. First, screening for coregulators has, for the most part, been limited to proteins that interact with the C-terminal ligand-binding domain of the receptors at activation function 2. However, activation function 1, located in the N terminus of the receptors, may be as important, if not more important, for the agonist properties of steroid antagonists, but this region has received little attention as a target for coregulatory protein binding. Newer experimental strategies may correct this deficiency [46,47].

Second, recent crystallographic analyses of steroid receptor ligand binding domains show that there are subtle structural variations in the conformation of receptors resulting from the binding of different ligands [48,49]. This would yield multiple, subtly different targets on the C-terminal surface of the receptors for the binding of a variety of coregulators, dictated specifically by the identity of the ligand. As discussed above, few, if any, ligand-specific coregulators have yet been identified, perhaps because multiple ligands have not been compared in screening assays. Third, while C-terminal receptor structure has been analyzed in detail, little is known about the structure of steroid receptor N termini. Yet, to judge from PR [50], this region

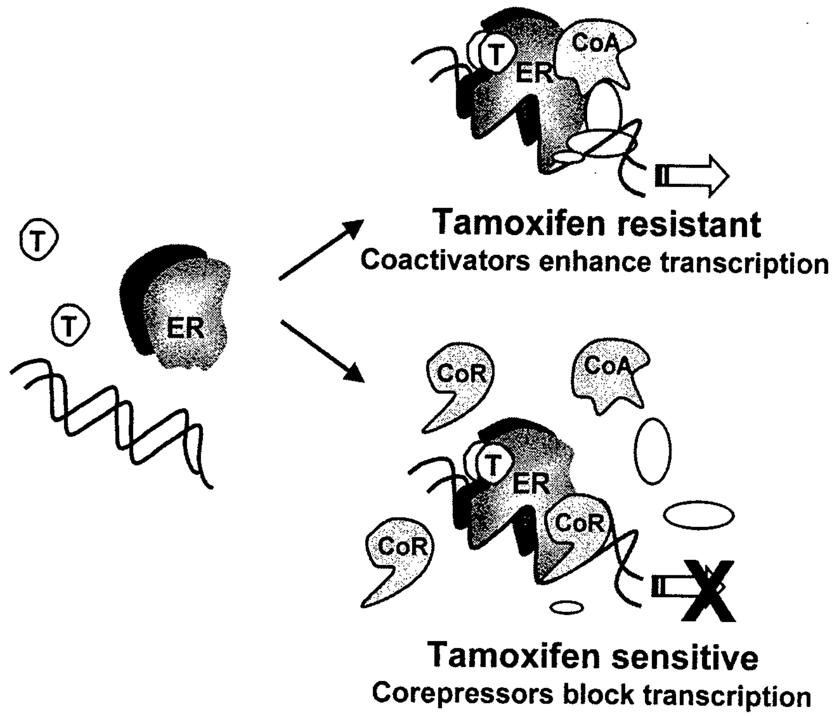

Fig. 1. Model for tamoxifen resistance. Tamoxifen (T) induces ER to undergo a conformational change that targets the receptor for interaction with both coactivators (CoA) and corepressors (CoR). Unopposed coactivators enhance transcription at specific loci, leading to an increased agonist activity and tamoxifen resistant phenotype. The increased corepressor level blocks transcription and maintains the tamoxifen-sensitive phenotype.

has an ordered but asymmetric structure that would offer multiple potential target sites for protein–protein interactions. The structure of steroid receptor N termini appears to be influenced by the DNA binding domain, and possibly also by DNA binding-induced allostery. Putative recruitment of coregulatory proteins could therefore occur either through induced fold mechanisms, generated by direct contact between the coregulators and the receptors at various sites along the N terminus, or through structural alterations in the N terminus (followed by binding of different coregulators) induced by DNA binding.

The latter, in particular, suggests a scenario in which different coregulators interact with receptors, depending on the gene to which the receptors are bound. Existence of such heterogeneity is suggested by the known tissue specificity of antagonists. For example, the usefulness of tamoxifen in breast cancer is due to its predominantly antagonist nature in the breast. However, in the uterus it is a potent estrogen, where, like estradiol (when unopposed by progesterone), it can induce epithelial hyperplasia and endometrial cancers. Are tamoxifen's mixed agonist/antagonist properties due to 1) activation of different sets of genes in the breast versus uterus; 2) the presence of different sets of coregulators in each tissue; 3) varying and limiting levels of coregulator subsets in each tissue; or 4) differential utilization of a common set of coregulators whose recruitment to the receptors is controlled by the identity of the ligand and by variable DNA structure at different gene loci?

Fig. 1 presents a modification of our initial working model, based on our recent experimental data, whereby tamoxifen induces a distinct conformational change in ER, transforming the receptor into a target of both coactivators and corepressors. In the absence of corepressors, unopposed coactivators bind and direct tamoxifen-liganded ER to specific gene targets, enhancing the activity of the basal transcriptional unit. However, sufficient corepressor levels are able to override this effect and block transcription, thus maintaining the antagonist properties of the ligand. If indeed, receptor conformation dictates the nature of the coregulators recruited to the transcription complex, it follows that these will differ for each ligand, minimizing the possibilites of cross-resistance. Is that why pure antiestrogens continue to inhibit tumors in tamoxifen-resistant patients?

Clearly, the answers to the above questions have important implications for our understanding of acquired tamoxifen resistance in breast cancer. Mechanism 1 above is likely to be the case, as gene array technology is beginning to show. On the other hand, to date, no tissue-specific coregulators have been described. Quite the contrary, all known coregulators are "ubiquitous." This suggests that mechanism 2 may not be critically important, and, if anything, that 3, which addresses the relative levels of coregulators, is more important. However, this scenario does not address the redundancy issue. Namely, the possibility that one coregulator can functionally replace another. Coregulator redundancy is suggested by SRC-1 knockout studies, which generated only subtle phenotypes in the SRC-1-deficient mice [51]. If the same coregulator complement and redundancy exists in breast cancers, it will, we believe, make it very difficult to correlate tamoxifen resistance with coregulator levels. That leaves scenario 4 above as a likely player in tamoxifen resistance—an extraordinarily complex one.

Acknowledgments

Studies from the authors' laboratories were supported by grants from the National Institutes of Health and the National Foundation for Cancer Research. Dr. Graham is supported by grants from the United States Army Breast Cancer Program and the Komen Foundation. A similar chapter has been submitted for publication of the proceedings of Nobel Symposium No. 113, held in Karlskoga, Sweden June 29–July1, 1999.

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